

c7
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at least a second position in a modified, dual-labeled modified Crk-II polypeptide, comprising the steps of:

- (a) providing a modified, dual-labeled Crk II molecule such as the Rh-(Crk-II)-Fl construct of Figure 5A and SEQ ID No:8;
- (b) subjecting the dual-labeled molecule to conditions inducing the activity in the presence and absence of the agent;
- (c) measuring the changes in relative proximity of the first and second interacting proximity-sensor peptides in the composition in the presence and absence of the agent; and
- (d) identifying an agent affecting the changes as capable of modulating the activity.--

At page 11, line 6 of the specification, please replace the existing paragraph with the following:

c3

Figure 1. Biosensor for c-Abl phosphorylation of the Crk-II adapter protein. c-Abl phosphorylates Crk-II on Tyr 221 which is thought to induce an intramolecular association with the SH2 domain. This rearrangement is expected to yield a net change in the distance between the termini of the protein, which would be reported by a dual-labeled derivative of Crk-II in which the FRET pair tetramethylrhodamine (Rh) and fluorescein (Fl) are specifically incorporated at its N- and C-termini, respectively.--

At page 19, line 1 of the specification, please replace the existing paragraph with the following:

c4

--An object of the present invention is to provide a generally accessible methodology which allows several recombinant and

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synthetic polypeptides to be regioselectively linked together, thereby allowing multiple different chemical probes to be site-specifically incorporated into the resulting semi-synthetic protein product. Proteins undergo conformational changes related to their activity or modified state, such as protein targets of phosphorylation and dephosphorylation. By use of probes which are environmentally sensitive, for example, those which are proximity sensitive, changes in their interaction may be monitored to identify the activity or state of the polypeptide. Thus, with a target of biological activity capable of reporting its activity and facile detection of the activity, the target is useful for several purposes. One such purpose is in identifying modulators of the interaction between the target and a molecule which affects the activity or biological state of the target. By way of non-limiting example, which will be exemplified in the Examples below, agents capable of modulating protein kinase activity may be identified using the constructs and methods herein. For example, a protein kinase and its target protein, the latter provided as a semisynthetic construct of the invention labeled to report the state and kinetics of phosphorylation is used. Under appropriate conditions, the combination of the protein kinase and the labeled target reports the protein kinase activity. By carrying out the measurement of the protein kinase activity in this manner in the presence and absence of a candidate agent for modulating protein kinase activity, one may identify inhibitors or activators of the protein kinase. Moreover, the inhibitors or activators may act on the protein kinase, or on the substrate, or both; further studies may be performed to identify the site of interaction. Agents capable of modulating the kinetics of enzymatic activity are detectable using these methods.--

At page 22, line 10 of the specification, please replace the

existing paragraph with the following:

C5 --EPL has been extended to permit the insertion of a synthetic peptide into a recombinant protein through the sequential ligation *in solution* of two recombinant protein fragments to the N- and C-termini of a synthetic peptide cassette (17). While this strategy is, theoretically, extendible to the ligation of any combination of synthetic and/or recombinant fragments, the need to perform all of the steps *in solution* renders the approach technically demanding; after each ligation reaction it is necessary to isolate the desired product from the reaction mixture, a process which is time-consuming and, importantly, leads to substantial handling losses. In principle, these problems should be overcome by transferring the entire process to the solid-phase, in a manner analogous to solid-phase peptide synthesis (SPPS) (18). As with SPPS, this solid-phase protein ligation (SPPL) approach should allow each reaction to be driven to completion by using a large excess of reagents, which can then be simply removed by washing. In addition, there would be no need to isolate intermediate ligation products which would remain immobilized on the support. The present inventors have developed an SPPL technology and have successfully applied it to the generation of a dual-labeled version of the ~35 kDa adapter protein, Crk-II. As is shown herein, this semi-synthetic protein analog specifically biosenses a post-translational tyrosine phosphorylation event important in regulation of Crk-II mediated signal transduction. Thus, it may be used for various purposes, such as to identify agents capable of modulating phosphorylation activity. It also provides an example for the design of other protein kinase targets, and more broadly, other useful polypeptides wherein biosensing conformational changes provides a useful tool for screening purposes and the like, as noted below.

At page 23, line 8 of the specification, please replace the existing paragraph with the following:

C6
--Various polypeptides which undergo conformational changes upon post-translational modification or other effects are candidates for the preparation of a semi-synthetic multiple labeled polypeptide constructs of the invention. Proteins which are themselves targets of enzymatic modification are preferred examples; targets of protein kinase activity are particularly preferred. Non-limiting examples of such targets include transcription factors and signal transduction factors. Numerous other targets are embraced herein, such as those reviewed in (35). In a most preferred embodiment, the polypeptide is an adapter protein. In a more preferred embodiment, the target is a target of the protein kinase c-Abl, such as Crk-II. Figure 1 illustrates the conformational change which the adapter protein undergoes on phosphorylation, and the change in proximity of a dual-labeled composition of the invention comprising the Crk-II polypeptide. The polypeptide of the invention may comprise the sequence of the entire target protein, or may comprise a fragment of the sequence, the fragment which comprises the site of the post-translational modification and the portions of the polypeptide which undergo the conformational changes to be measured in an aspect of the instant invention. Various modifications which do not detract from the utility of the fragment may be made, for example, to facilitate ligation to the sensor peptides, expression, optimal placement of the sensor peptides, and ease of synthesis or purification, among others.--

At page 24, line 1 of the specification, please replace the existing paragraph with the following:

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--Two or more probes may be provided in the semi-synthetic polypeptide. Such probes are selected to report their relative proximities. For example, fluorescence resonance energy transfer (FRET) pairs provide a fluorescence reading depending on the proximity of the fluorophores. For example, fluorescein and tetramethylrhodamine may be used. Other pairs include IAEDANS and fluorescein, EDANS and DABCYL, BODIPY FL fluorescein and BODIPY fluorescein, β -phycoerythrin and CY5, and pyrene and coumarin. FRET pairs are known in the art and a skilled artisan can readily select appropriate pairs for use in the compositions of the invention. The probes of the invention are modified peptides in which the fluorophore or other reporter moiety is provided as a side chain or in the polypeptide backbone. Examples include Dapa-fluorescein (diaminopropionic acid-fluorescein) and N^α -tetramethylrhodamine-KRG. Others include peptides or oligopeptides with a moiety, such as EDANS, IAEDANS, DABCYL, BODIPY fluorescein, β -phycoerythrin, CY5, pyrene, or coumarin, capable of participating as a FRET pair with another modified oligopeptide. As noted in the examples herein, which are not limiting, the labeled peptides are provided in forms to be incorporated in a stepwise fashion into the dual-labeled polypeptide. In one synthetic strategy, as described in the Examples below, the labeled peptides may be provided in a form for eventual enzymatic or chemical cleavage to, for example, release the product from a substrate. Thus, the reactants may have cleavage sites therein to facilitate synthesis. In an example herein, shown in Figure 1, Crk-II (adapter protein; phosphorylation target of c-Abl) is recombinantly expressed as a fusion construct at the N-terminus of an intein-chitin binding domain (Xa-Cys-(Crk-II)-Intein-CBD). An N-terminal cysteine is included to facilitate ligation. The recombinant construct is bound to chitin beads through the chitin-binding domain. In the first step, the above construct is reacted

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with CGK(F1)-GLEVFQGPVRKGK(Biotin)GNH₂ ("Cys-F1-PS-Biotin"; SEQ ID No:6), wherein the N-terminal cysteine is ligated to the Crk-II, forming the product Xa-Cys-(Crk-II)-F1-PS-Biotin. The ligated product is then bound to avidin beads through the biotin moiety on the C-terminal portion of the fluorescein-labeled peptide. The Xa portion is then cleaved with factor Xa, and the now exposed N-terminal cysteine reacted with N^α-tetramethylrhodamine-KRG-propionamide thioester to ligate the cysteine with the thioester. Subsequently, the PS peptide is cleaved, yielding the dual-labeled product.--

At page 25, line 12 of the specification, please replace the existing paragraph with the following:

C8

--The present invention is directed to the semi-synthetic constructs comprising a target polypeptide and multiple probes, as well as methods for using these constructs in monitoring the biological activity of the polypeptide upon modification (or return to its native state) as well as its use in identifying agents capable of modulating the modification. Numerous examples of polypeptides that are targets of post-translational and other modifications, especially reversible modifications, are available. By way of non-limiting example, targets of protein kinase activity are preferred embodiments of the present invention. Such include signal transduction factors and transcription factors, as non-limiting examples, as further exemplified in (35). Protein kinases and their phosphorylation/dephosphorylation targets are implicated in critical pathways in which perturbations are known to lead to clinically significant derangements, such as cellular transformation and carcinogenesis. In particular, the protein kinase c-Abl and its target Crk-II are involved in cellular regulation, derangements of which can lead to cellular dysfunctions. Identification of molecules capable of

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Crk-II*

preventing phosphorylation of Crk-II are candidates for pharmaceutical development. Heretofore, assays of compounds for modulation of phosphorylation required the use of ^{32}P and critical measurements of labelling of target molecules. The instant invention provides a facile means to identify modulators of phosphorylation by monitoring changes in the interaction of multiple labels on the phosphorylation target, induced by changes in confirmation consequent to phosphorylation. Rapid, automated high-throughput screening of compounds may be performed using the constructs and methods of the present invention.--

At page 27, line 1 of the specification, please replace the existing paragraph with the following:

C9

--As will be seen in the Examples below, the synthesis was carried out of a semi-synthetic version of the adapter protein, Crk-II, in which the FRET pair, tetramethylrhodamine and fluorescein were incorporated at the N- and C-termini of the protein, respectively (hereafter referred to as Rh-(Crk-II)-Fl), as described in summary above. Crk-II has been implicated in a number of cellular signaling processes, and is composed predominantly of one Src homology 2 (SH2) and two SH3 domains through which it mediates intermolecular protein-protein interactions (22, 23). Two protein tyrosine kinases, c-Abl and the epidermal growth factor receptor (EGFR), are known to phosphorylate Crk-II on a unique tyrosine residue (Tyr221) located between the SH3 domains (24, 25). This post-translational modification is thought to regulate Crk-II function by inducing an intramolecular association with the SH2 domain (26) which in turn inhibits certain intermolecular protein interactions (22-25). It was anticipated that phosphorylation and subsequent intramolecular association would result in a distance change between the termini of Crk-

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II, which would lead to a change in FRET between the two fluorophores in the dual-labeled analog (Fig. 1). Consequently, this protein construct would directly biosense this important post-translational event.--

At page 27, line 19 of the specification, please replace the existing paragraph with the following:

c10

--The preparation of the construct Rh-(Crk-II)-Fl is summarized in Fig. 2A. As with SPPS, the strategy can be divided essentially into three parts; attachment of the first building block to a solid support (e.g., avidin beads), chain assembly in a C-to-N direction involving successive deprotection and ligation steps, and cleavage of the completed polypeptide off the solid support. In the first step, full length mouse Crk-II was expressed as an in-frame fusion to an engineered yeast VMA intein which allows the subsequent generation of a reactive ^athioester derivative of Crk-II. In this example, an extra Gly residue was added to the C-terminus of Crk-II to improve the kinetics of the first ligation reaction (8), and the N-terminal Met was replaced by the sequence -IEGRC (Xa-Cys) (SEQ ID NO:10) to facilitate controlled sequential ligation (17). Soluble expression of this fusion protein [Xa-Cys-(Crk-II)-Intein-CBD] was optimized using standard protocols (no *in vivo* intein cleavage of the full length fusion could be detected) and the desired material purified by affinity chromatography using a chitin column.--

At page 31, line 1 of the specification, please replace the existing paragraph with the following:

c11

--As will be shown in a further Example, below, phosphorylation studies were performed on the construct to demonstrate its utility in identifying modulators of protein

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kinase activity. Purified Rh-(Crk-II)-Fl was assayed for its ability to biosense Crk-II phosphorylation by the c-Abl protein tyrosine kinase. As indicated previously, phosphorylation by c-Abl leads to an intramolecular association between a phosphotyrosine motif and the Crk-II SH2 domain, which can be reported by the dual-labeled Crk-II derivative (Fig. 1). Rh-(Crk-II)-Fl was treated with full length recombinant c-Abl and aliquots of the reaction mixture were analyzed by fluorescence spectroscopy and western blotting at ~1 min and 60 min time-points. In the absence of ATP, essentially no change in FRET (i.e. the ratio of the fluorescein/tetramethylrhodamine emission intensities) was observed during the reaction (Fig. 3A), and no Rh-(Crk-II)-Fl phosphorylation could be detected using an anti-phosphotyrosine monoclonal antibody (Fig. 3B). In contrast, when ATP was included in the reaction mixture, a phosphorylation-dependent increase in the emission intensity ratio (a decrease in FRET) was consistently observed. Rh-(Crk-II)-Fl was completely phosphorylated after 1 h as determined by native PAGE mobility (Fig. 3C). The quite modest decrease in FRET (~3% after 60 min) suggests that the SH2-phosphotyrosine interaction, which is triggered by Rh-(Crk-II)-Fl phosphorylation, results in only a small net change in the relative distance between the N- and C-termini in the protein.--

At page 36, line 18 of the specification, please replace the existing paragraph with the following:

--The resonance energy transfer between the fluorophores in the unphosphorylated molecule was calculated to be 52.5% as determined from both the quenching of the fluorescein emission intensity and the sensitized emission of the rhodamine acceptor (as in ref. 34). Assuming that both fluorophores have

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random orientations and using a Förster distance of 45 Å for the F1-Rh pair (34), then the distance between the two fluorophores is ~44 Å. Interestingly, this suggests that unphosphorylated Crk-II has a somewhat compacted domain architecture, as opposed to a linear array of domains; based on the primary sequence, the N- and C-termini could be as much as ~200 Å apart if the inter-domain linkers assume a fully extended conformation.--

At page 42, line 18 of the specification, please replace the existing paragraph with the following:

C13

--In the first step, full length mouse Crk-II was expressed as an in-frame fusion to an engineered yeast VMA intein which allows the subsequent generation of a reactive thioester derivative of Crk-II. An extra Gly residue was added to the C-terminus of Crk-II to improve the kinetics of the first ligation reaction (8), and the N-terminal Met was replaced by the sequence -IEGRC (Xa-Cys) (SEQ ID NO: 10) to facilitate controlled sequential ligation (17). Soluble expression of this fusion protein [Xa-Cys-(Crk-II)-Intein-CBD] was optimized using standard protocols (no *in vivo* intein cleavage of the full length fusion could be detected) and the desired material purified by affinity chromatography using a chitin column.--

At page 43, line 6 of the specification, please replace the existing paragraph with the following:

C14

--A synthetic peptide, Cys-F1-PS-Biotin, containing both a fluorescein probe (F1) and a biotin affinity handle separated by a linker region containing the cleavage site for the PreScission protease [LEVLFQGP, (PS)], (SEQ ID NO: 1) was chemoselectively ligated to the C-terminus of recombinant Crk-

c19
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II using EPL. This ligation reaction was found to be <95% complete after 48h in the presence of a large excess of peptide and the thiol cofactors ethanethiol and MESNA. Gel filtration was used to separate the unreacted peptide from the desired ligation product which was then attached to monomeric-avidin beads via its biotin functionality. Preliminary model studies had established that the monomeric-avidin-biotin complex was stable to all the washing, deprotection and ligation steps used in SPPL, but that the interaction can be disrupted under mild conditions with exogenous biotin. Trace amounts of unreacted Crk-II protein and any remaining bacterial protein contaminants were then removed by vigorously washing the beads with high salt and detergent at pH 5.2 and pH 8.0. This yielded the pure protein, Xa-Cys-(Crk-II)-F1-PS-Biotin, immobilized on a solid-support (Fig. 2B, Lane 2).--

In the claims:

Please add new claims 51-77, as follows:

C15
51. A composition for detecting the effect of an enzyme on a substrate, comprising an active enzyme peptide or functional fragment thereof which acts on a peptide substrate, said enzyme activity producing a modification of at least one substrate amino acid, said substrate remaining intact, and a peptide substrate or functional fragment thereof for said enzyme, wherein at least one, but not both of said active enzyme peptide and said peptide substrate comprises at least a first detectable proximity-sensor peptide incorporated into a first position of one of said peptides and a second detectable proximity-sensor peptide incorporated into a second position of said one peptide, thereby providing a semi-synthetic multiple labeled polypeptide, wherein said semi-synthetic multiple labeled polypeptide has a first structural conformation in an unmodified state and a second structural conformation in a modified state, said proximity sensors being